METABOLIC ANTAGONISTS AND PROLONGED SURVIVAL OF SCALE HOMOGRAFTS IN FUNDULUS HETEROCLITUS[1]

RICHARD J. GOSS[2]

Department of Biology, Brown University, Providence, R. I., and Mount Desert Island Biological Laboratory, Salisbury Cove, Maine

The immunological competence of an animal depends upon its ability to synthesize antibodies, the specificity of which appears to be regulated by nucleic acids. The exact mechanism whereby antigenic information is translated into antibody specificity in the course of protein synthesis, however, remains more speculated about than understood. In order to investigate this, attempts have been made to interrupt the hypothetical pathway along which such information might be expected to be transferred. Accordingly, the syntheses of nucleic acids and proteins have been interfered with by means of various antimetabolites, and the subsequent capacity for antibody formation studied.

As an experimental system, the homograft reaction against foreign scale transplants in *Fundulus heteroclitus* has been adopted. The usefulness of this technique in analyzing the homograft reaction has been demonstrated by Hildemann (1956, 1957a, 1957b, 1958) and Hildemann and Haas (1960). These investigators recognized the rejection of foreign scale grafts as a temperature-dependent inflammatory response leading to the destruction of the graft in 4.3 days at 32° C. to 40.5 days at 10° C. A further innovation was recently reported by Triplett and Barrymore (1960) who utilized the time of disintegration of melanophores on transplanted scales as the criterion for estimating the time of onset of the homograft reaction. According to this method, foreign scale grafts survived an average of 5.8 days at 20° C. and 7.0 days at 17° C. This technique of observing the duration of melanophore survival after grafting has made it possible to judge easily, quickly, and accurately the time required for the host to react against the presence of immunogenically foreign cells. The relatively abrupt nature of the overt response represents a distinct advantage over the more subjective methods of estimating homograft survival times in higher vertebrates.

There is a measure of uncertainty concerning the exact nature of the homograft response, particularly as regards the cellular vs. humoral location of factors alleged to be responsible for reaction against foreign tissues (*cf.* Brent, Brown and Medawar, 1959; Lawrence, 1960; Gorer, 1960). The demonstration by Triplett and Barrymore (1960) that homograft sensitivity can be transferred via intraovarian fluid from pregnant females to their embryos argues in favor of the possible existence of circulating antibodies, at least in fish. Since the present account, unhappily, cannot further resolve this problem, the author elects to assume that

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[2] Fellow of the Carnegie Institution of Washington (Department of Embryology), 1960–61.

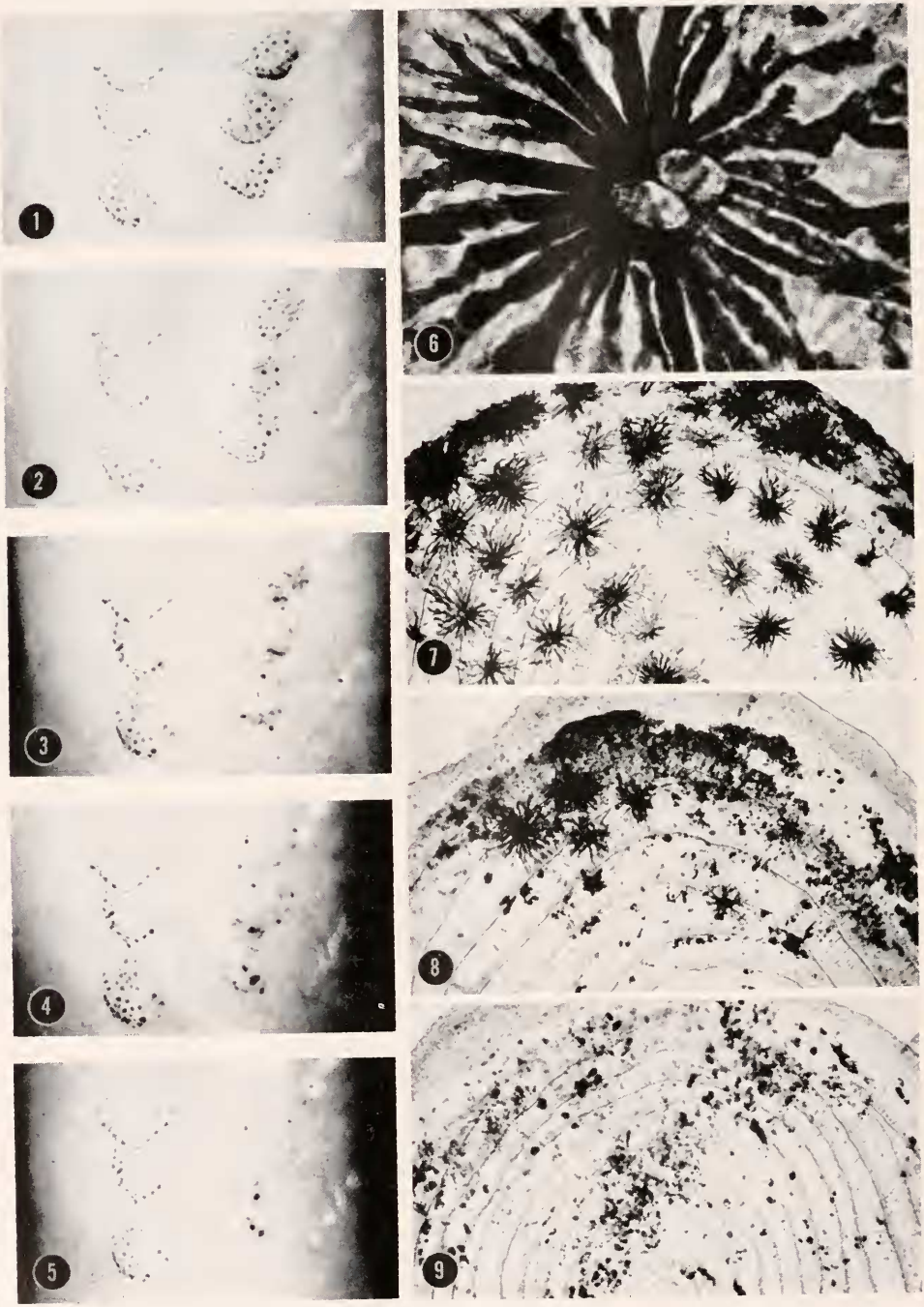
the rejection of homografts is an immunological phenomenon attributable to antibodies, without reference to their disposition or mode of action, but very much concerned with their genesis. As assayed by the prolonged survival of scale homografts on treated hosts, it has been found in the present experiments that under conditions of restricted protein or nucleic acid synthesis, antibody production has been inhibited.

Materials and Methods

Experiments were performed on male *Fundulus heteroclitus* weighing an average of 6 to 8 grams each. Fish were maintained in running salt water aquaria at a temperature of $28 \pm 1°$ C. except where otherwise stated. All operations were performed on fish under chloretone (1 : 1000) anesthesia. Scale transplantations were achieved by inserting scales in the dermal scale pockets from which the original scales had been plucked. If care is taken to graft scales of the same size as those being replaced, the transplants are rarely lost. All transplants were made to the unpigmented ventral region of the fish. In each animal three autografts and three homografts were inserted in parallel rows on either side of the linea alba. Pigmented scales derived from the posterior region of the fish proved to be of a size commensurate with those of the ventral transplantation site. Each experiment comprised four fish in which homografts were made reciprocally between pairs.

Circulation in the scale graft was re-established on the day following operation, at which time there was usually observed a slight nonspecific degeneration of a few pigment cells in both autografts and homografts (attributable to injury attending the transplantation procedures). Animals bearing scale grafts were examined daily under the dissecting microscope to determine the condition of melanophores in the transplants. In control fish, foreign melanophore breakdown was invariably complete on the third day after grafting at 28° C. (Figs. 1–5). The reliability of this was such that the survival of pigment cells only one day beyond this time constituted an unequivocal and statistically significant indication of the efficacy of the treatment being tested. The number of days designated as the survival time refers to the day on which complete or very extensive destruction of pigment cells was observed to occur. As a record of the experiments, photomicrographs were taken through the dissecting microscope of scale transplants at critical times.

In addition to the effects of temperature change, splenectomy, hypophysectomy, and trypan blue, the following substances, occasionally in varying doses, were administered to hosts by daily intraperitoneal injection in 0.1 ml. distilled water. Materials which interfere with nucleic acid synthesis were 5-fluorouracil, 5-fluorodeoxyuridine (FUDR) and 6-mercaptopurine (6-MP). Adrenal cortical hormones included cortisone acetate, 6-fluorohydrocortisone acetate and delta-1-hydrocortisone sodium succinate (Delta-Cortef: The Upjohn Co.). Two antibiotics were tested: chloramphenicol sodium succinate (Chloromycetin: Parke, Davis & Co.) and tetracycline hydrochloride (Achromycin: Lederle). The amino acid analogues used were β-2-thienylserine and DL-β-phenylserine (serine analogues), DL-α-CH_3 phenylalanine, β-2-thienylalanine and DL-β-phenyllactic acid (phenylalanine analogues), and ethionine (methionine analogue).



FIGURES 1–5. Daily photographic sequence of autograft (left) and homograft (right) scales on the first through fifth days after transplantation at 28° C. The row of three homo-

Results

Temperature effect

Groups of fish each bearing autografts and homografts were maintained at temperatures of 7°, 14°, 21°, and 28° C. All except the 7° C. group, which was kept in a refrigerator, were in circulating sea water. The scale grafts were inspected daily and photographed at frequent intervals to determine as precisely as possible the time of melanophore fragmentation. The lower the temperature the greater variation there was in the end point. At 28° C., incipient breakdown of pigment cells was detectable two days after operation, but not until the third day had all melanophores been destroyed. At this temperature, the reaction is relatively abrupt. Homotransplants of fish at 21° C. underwent pigment cell disintegration on the fifth and sixth days after grafting. Those maintained at 14° C. required 14 to 16 days to break down. At 7° C. the pigment cells of the homografts remained intact for 26 days at which time it was necessary to terminate the experiment. In all groups of fish, the autograft scales remained healthy indefinitely.

Splenectomy

In four fish, the spleens were removed via a ventral incision on the day prior to scale grafting. Four controls were subjected to sham operations. In all fish, controls and experimentals, the homografts broke down on the third day. The results are consistent with those of Vogel (1940) who noted that splenectomy failed to protect skin homografts in *Rana pipiens* from destruction.

Hypophysectomy

Animals were deprived of their pituitaries, or subjected to sham operations, two days before scales were grafted. This operation did not enhance the survival of homografts. In 12 hypophysectomized fish and 13 controls, the homografts exhibited breakdown of pigment cells on the third day.

Trypan blue

Four experimental animals received scale grafts on the day of the first intraperitoneal injection of 0.1 ml. of 1% trypan blue in distilled water. Injections were repeated daily. Control fish similarly grafted were injected intraperitoneally with 0.1 ml. distilled water daily. On the third day there was complete breakdown of pigment cells in the homografts of both control and experimental groups, despite the fact that the treated fish had become intensely stained with dye.

Nucleic acid antagonists

The injection of substances which inhibit nucleic acid synthesis proved to be very successful in protecting the homograft scales from the antibody response of

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graft scales exhibited pigment cell breakdown on the third day, with subsequent disappearance of the pigment granules. Autografts remained intact throughout. 10 ×.

Figure 6. A normal, expanded, scale melanophore showing typical binucleate condition. 1000 ×.

Figures 7–9. Appearance of scales before, during and after onset of homograft reaction. 100 ×.

the host. In two separate experimental series in which a total of eight fish were given 1 mg. of 5-fluorouracil each daily, starting on the day of operation, the homografts showed no signs of pigment cell breakdown as long as the fish survived. This dose, although effective, was at the same time toxic and resulted in the deaths of animals after two to six days. Nevertheless, four fish still alive on the fifth day possessed intact scale homografts, and it would appear that their transplants would have survived longer had the hosts lived. Similar experimental series on eight fish injected with 2 mg. of 5-fluorodeoxyuridine (FUDR) likewise resulted in protection of the homografts for up to eight days, by which time five of the animals had died as a result of the toxic effects of the drug. Injection of 2 mg. of 6-mercaptopurine into each of four fish bearing homograft scales afforded protection for four days at which time the injections were discontinued. Thereafter, melanophores were gradually destroyed until only one intact homograft scale remained on the seventh day, when the experiment was terminated. From these results it is clear that near-lethal doses of these drugs effectively prolong the survival time of homografts. In the cases of all three substances, when the doses were reduced to 1/100 of the above levels, no protection whatever was observed.

Adrenal cortical hormones

Daily intraperitoneal injection of a 1 mg. suspension of cortisone acetate to grafted fish starting on the day of operation had no beneficial effect on the survival of the homografts. On the third day there was complete breakdown of all scale pigment cells, probably due to inadequate doses of cortisone. More potent preparations of cortical hormones, however, proved to be more effective. Injections of 2 mg. of 6-fluorohydrocortisone actetate on the day of transplantation and on the two days thereafter resulted in the survival of homografts for four days, at which time about half of all the pigment cells were undergoing fragmentation. This nevertheless marks a definite delay in the destruction of the grafts. In a third experiment, 2 mg. of delta-1-hydrocortisone sodium succinate were injected daily through the second day after operation. In these fish, no pigment cell breakdown was observed on the third day, an incipient destruction was noted on the fourth, and, after five days, disintegration was well progressed in all except one animal in which the homografts remained intact. This compound, therefore, exerted a distinct protective effect.

Antibiotics

Chloramphenicol sodium succinate was tested at two dose levels. Daily injection of 1 mg. per animal intraperitoneally had no detectable effect on the survival time of the homografts. Similar injections of 10 mg. chloramphenicol, however, resulted in survival of homograft pigment cells beyond the third day. On the fourth day there was breakdown of melanophores in the grafts of one fish, and on the next day extensive, but still not complete, disintegration of foreign pigment cells had occurred. All were destroyed by the sixth day.

Tetracycline hydrochloride was likewise tested at two different dosages. Injection of 0.1 mg. per fish gave no protective effect. Administration of 1 mg. of tetracycline per day through the third day resulted in prolonged survival of homograft

pigment cells. In three fish the grafts were destroyed on the fourth day, and in three others they broke down on the fifth day. In two animals the pigment cells of homograft scales were still intact on the seventh day when the experiment was terminated. These antibiotics, therefore, interfere with the immunological response of the host against foreign grafts.

Amino acid analogues

Six different analogues of amino acids were tested for possible interferences with the homograft reaction. In general, they proved to be rather toxic and not very effective in protecting the foreign scale grafts from destruction by the host. In all cases, at least two dose levels were tried, the larger usually representing the limits of solubility in 0.1 ml. distilled water. Two analogues of serine were

TABLE I

Summary of effects of antimetabolites, administered to hosts, on survival of scale homografts at 28° C.

Substance injected (IP)	Dose (mg./fish/day)	Survival time (days)
Controls	—	3
5-fluorouracil	0.01	3
	1.0*	5+
5-fluorodeoxyuridine	0.02	3
	2.0*	6–8
6-mercaptopurine	0.2	3
	2.0	5–7
Cortisone	1.0	3
6-fluorohydrocortisone	2.0	4–5
Delta-1-hydrocortisone	2.0	5
Chloramphenicol	1.0	3
	10.0	4–6
Tetracycline	0.1	3
	1.0	4–7
β-2-thienylserine	1.0	3
	5.0	3–4
DL-β-phenylserine	1.0	3
	5.0*	3–4
DL-α-CH_3 phenylserine	1.0	3–4
	3.3	3–4
β-2-thienylalanine	1.0	3
	5.0	4
DL-β-phenyllactic acid	1.0	3
	2.5*	3
	5.0*	4
	10.0*	—
Ethionine	1.0*	3–4
	3.3*	5

* Lethal dose.

used. β-2-thienylserine and DL-β-phenylserine were injected in doses of 1 mg. and 5 mg. into groups of four fish. The smaller dose in all cases was ineffectual; the larger dose resulted in survival of homograft pigment cells for four days. None of these doses was lethal except in one fish which died on the third day following injections with 5 mg. of DL-β-phenylserine. Analogues of phenylalanine included DL-α-CH_3 phenylalanine which was administered in doses of 1 mg. and 3.3 mg. per day to groups of four fish. In all cases, foreign pigment cell breakdown was initiated on the third day but was not complete until the fourth. There was no detectable difference between the effects of the two doses utilized, nor did either dose prove to be lethal. β-2-thienylalanine was injected in doses of 1 mg. and 5 mg. daily. Only the larger dose prolonged the survival of scale homografts (to the fourth day). DL-β-phenyllactic acid, also an analogue of phenylalanine, was given in four different doses to four groups of fish. Doses of 1 mg. and 2.5 mg. failed to protect the homografts from destruction on the third day. A single injection of 5 mg. to another group of fish on the day of transplantation killed two fish the next day, but enabled the homografts of the remaining two animals to survive to the fourth day before being destroyed. Administration of 10 mg. of this compound proved lethal to all fish within one day. A final analogue, ethionine, was tested at levels of 1 mg. and 3.3 mg. The lesser dose permitted homografts to survive until the fourth day; 3.3 mg. per day through the second day after transplantation resulted in survival of homografts for two days beyond the controls. The latter dose, however, was lethal to three out of four fish by the fourth day after operation, at which time the foreign pigment cells were still intact. The one fish still alive on the fifth day exhibited complete breakdown of its homograft melanophores at that time. Ethionine therefore was considerably more effective in enhancing the survival of homografts than were the other five amino acid analogues tested.

Discussion

The immunological reaction leading to homograft destruction has been divided into three phases (Billingham, Brent and Medawar, 1956), involving the release of graft antigens (afferent phase), the production of antibodies (central phase), and the reaction of antibodies with the graft (efferent phase). Although interruption at any one of these levels would insure homograft survival, it is the central phase which is most amenable to experimentation. The process of antibody production may in turn be partitioned into subsidiary processes, leading from the initiating influence of the antigen on antibody-producing cells (induction or adaptation phase) to the eventual fabrication of antigen-specific antibodies (production phase). The method by which antibodies are formed is essentially a problem of protein synthesis with the added prerequisite that the protein antibody be capable of reacting specifically with the antigen originally responsible for initiating its formation. The synthesis of such specific proteins necessarily involves the participation of a system by which the nature of the specificity can be communicated from the antigen to the molecular architecture of the antibody concomitant with its synthesis. There is reason to believe that nucleic acids constitute such a communication system. This is substantiated by the dependent relationships of protein synthesis on RNA and of specific RNA synthesis on DNA. If this system is to remain sufficiently labile to adapt to new modes of protein synthesis (*e.g.*, specific

antigen-stimulated antibody production) it is necessary to assume that new specific types of RNA molecules can be synthesized on demand. This requirement may be taken to indicate that RNA synthesis is necessary for the production of specific proteins. Schweet and Owen (1957) have postulated that antigen reacts with DNA which in turn makes specific RNA, and that the RNA acts as template in giving rise to specific antibodies.

It is not surprising, therefore, that analogues of purines (6-MP) and pyrimidines (5-fluorouracil and 5-fluorodeoxyuridine), which interfere with nucleic acid synthesis, likewise arrest antibody production and thus result in the prolonged survival of homografts on treated hosts. Studies on 8-azaguanine, a purine analogue, have shown that it also inhibits nucleic acid synthesis (Skipper *et al.*, 1951) and antibody production (Malmgren, Bennison and McKinley, 1952; Dutton, Dutton and George, 1958). Berenbaum (1960) demonstrated that 6-MP also inhibits the production of antibodies, and Schwartz and Dameshek (1960) and Meeker *et al.* (1960) have reported the protection of skin homografts in rabbits by the administration of 6-MP.

It is generally acknowledged that many antibiotics exert their growth-limiting effects by inhibiting protein synthesis, either directly or indirectly. Chloramphenicol, for example, has been noted to prevent the synthesis of DNA (Drakulic and Errera, 1959; Schneider, Cassir and Chordikian, 1960), RNA (Gros and Gros, 1956; Webster, 1957), and protein (LePage, 1953; Smith, 1953; Pardee and Prestidge, 1956; Webster, 1957; Gale, 1958) in bacteria and mammalian tissues. Because of such manifold effects of chloramphenicol, and probably other antibiotics as well, their interference with antibody production and the homograft reaction is not unreasonable.

Amino acid analogues, in so far as they have been tested, were generally less effective in protecting homografts from destruction than were the other agents already discussed. There is evidence that β-2- and β-3-thienylalanine inhibit antibody formation in the rat (Ferger and du Vigneaud, 1949; Wissler *et al.*, 1956), and thymidine uptake in DNA is inhibited by β-2-thienylalanine and ethionine (Schneider, Cassir and Chordikian, 1960). Amino acid analogues are generally agreed (Matthews, 1958; Shive and Skinner, 1958) to act either by preventing protein synthesis via interference with the utilization of natural amino acids or by becoming incorporated themselves into proteins, thus displacing their normal counterparts. Of the amino acid analogues studied in the present investigation, at least ethionine and β-2-thienylalanine have been shown to act in the latter fashion (Levine and Tarver, 1957; Munro and Clark, 1958; Munier and Cohen, 1959). Structurally defective proteins would be expected not to be biologically inactive unless the incorporated analogues occupied an indispensable position. Since considerable portions of protein molecules are known to be functionally superfluous, the relative ineffectiveness of amino acid analogues in promoting homograft survival may find an explanation along these lines of reasoning.

The ability of cortisone to protect homografts from immunological destruction is too well known to require elaboration (Morgan, 1951; Billingham, Krohn and Medawar, 1951; Krohn, 1954; Medawar and Sparrow, 1956; Scothorne, 1956; Hamer and Krohn, 1959). This hormone also depresses antibody production (Germuth and Ottinger, 1950; Kass and Finland, 1953; Berglund, 1956) and

inhibits nucleic acid synthesis (Skipper *et al.*, 1951). It has been claimed that these effects of cortisone are augmented by its interference with the release of antigens during the afferent phase of the homograft reaction (Billingham, Krohn, and Medawar, 1951; Medawar and Sparrow, 1956; Scothorne, 1956). In view of the well documented evidence in favor of the efficacy of cortisone in suppressing the homograft reaction, plus the demonstrated effectiveness of the more potent preparations (6-fluorohydrocortisone and Delta-1-hydrocortisone), the failure of cortisone to enhance the survival of scale homografts in the present experiments may reasonably be ascribed to insufficient dosages.

With reference to the mode of action of the various agents found effective in promoting extended survival of homografts, it could be argued that such results might be attributed to nonspecific toxicities rather than to effects directly related to the inhibition of antibody synthesis. Although some of the drugs tested proved to be fatal at effective doses, there is little reason to conclude that their efficacy resulted directly from their lethality *per se*. The majority of the compounds which prolonged homograft survival manifested no other toxic effects during the period of treatment. Moreover, in the case of DL-β-phenyllactic acid, a dose of 2.5 mg. was lethal without being effective in precluding the homograft reaction. Additional treatments not reported here have also failed to interfere with foreign tissue rejection at otherwise lethal doses. Thus, while inhibition of nucleic acid or protein synthesis may be fatal, other kinds of toxicity need not interfere with immunological mechanisms.

The accumulated evidence supports the contention that homograft rejection may be subject to a moratorium in the absence of the successful synthesis of nucleic acids and/or proteins. On the basis of the limited number of compounds tested, there is reason to expect that numerous other agents with comparable physiological properties might exert similar influences. Granted that there are numerous factors which inhibit antibody production and thus actually or potentially interfere with the homograft reaction, it remains to be demonstrated conclusively whether such effects are permanent or temporary. In their investigations of the beneficial effects of 6-MP on skin homograft survival in rabbits, Meeker *et al.* (1960) noted that sustained treatment was necessary to insure continued survival of the grafts. In the present experiments, a comparable conclusion seems to be indicated, for despite the survival of scale homografts in treated hosts beyond the control period, eventual though dilatory breakdown was the rule. Notwithstanding these preliminary observations, it remains as a theoretical possibility that a specific tolerance might be conferred upon an adult host exposed to a foreign antigen by selectively inactivating those antibody-synthesizing pathways specifically stimulated by the antigen. If, as Burnet (1959) contends, antibody-producing clones are descended from specific cells stimulated to proliferate by exposure to antigen, then the application of treatments designed to render such cells vulnerable to destruction or inactivation at this critical period should, perforce, result in an animal subsequently tolerant to the original antigen. Alternatively, if antibody production can continue irrespective of whether or not the involved cells are stimulated to proliferate, specific tolerance could be realized only by permanently and selectively incapacitating the biochemical pathways by which the specifically stimulated antibodies are synthesized. To achieve this without doing violence to any other mechanism of protein synthesis will be a challenging enterprise.

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Summary

1. At 28° C., the melanocytes on scale homografts in *Fundulus* are destroyed in three days by the immunological response of the host. This reaction is slower to occur at progressively lower temperatures, but is not adversely affected by splenectomy or hypophysectomy of the host, nor by daily injections of trypan blue.

2. Survival of homografts was enhanced by daily intraperitoneal injections of base analogues, potent preparations of adrenal cortical hormones, antibiotics and amino acid analogues.

3. These results are taken to indicate that the inductive and productive phases of antibody formation are particularly vulnerable to agents which interfere with protein and/or nucleic acid synthesis.

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